APPENDIX B

Reduction of *O*-GlcNAc protein modification does not prevent insulin resistance in 3T3-L1 adipocytes

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Robinson KA, Ball LE, Buse MG. Reduction of *O*-GlcNAc protein modification does not prevent insulin resistance in 3T3-L1 adipocytes. *Am J Physiol Endocrinol Metab* 292: E884–E890, 2007. First published November 22, 2006; doi:10.1152/ajpendo.00569.2006.—3T3-L1 adipocytes develop insulin-resistant glucose transport upon preincubation with high (25 mM) glucose, provided that insulin (0.6 nM) is included, Akt activation is impaired, and high glucose and insulin act synergistically. Considerable evidence suggests that increased glucose flux via the hexosamine biosynthesis pathway enhances the *O*-Glc-NAc modification (*O*-GlcNAcylation) of some critical protein(s) that may contribute to insulin resistance. However, whether enhanced protein *O*-GlcNAcylation is necessary for the development of insulin resistance is unknown. We used two strategies to test this hypothesis. The first strategy was the overexpression of *O*-GlcNAcase, which removes *O*-GlcNAc from Ser/Thr of proteins. Cells were infected with *O*-GlcNAcase-expressing adenovirus (or empty virus) 5 days before they were submitted to protocols that elicit (or not) insulin resistance. *O*-GlcNAcase was highly expressed and functional as assessed by Western blot, *O*-GlcNAcase assay, and marked reduction of *O*-GlcNAcylated proteins. The activity was mainly cytosolic. The second strategy was the expression of *O*-GlcNAc transferase (OGT) being markedly reduced by transfection of OGT siRNA, resulting in an approximately 90% decrease of nuclear and cytosolic OGT protein expression and similar reduction in *O*-GlcNAcylated proteins. Non-targeting siRNA had no effect. Preincubation in high glucose with low-dose insulin decreased the acute insulin response of glucose transport by at least 50% and impaired Akt activation. None of these parameters were affected by overexpression of *O*-GlcNAcase or by OGT knockout. Excess *O*-GlcNAcylation is one of many factors that can cause insulin resistance. It does not seem to be required for the development of glucose/insulin-induced insulin resistance of glucose transport and Akt activation in 3T3-L1 adipocytes.

glucose transport; Akt activation; *O*-linked *N*-acetylglucosamine

INSULIN RESISTANCE IS A HALLMARK of type 2 diabetes and is associated with uncontrolled type 1 diabetes, obesity, and the metabolic syndrome, as well as numerous other conditions such as cystic fibrosis, polycystic ovary syndrome, uremia, septicemia, glucocorticoid excess, and others. Clinically, insulin resistance is defined as the decreased ability to lower plasma glucose in response to a given dose of insulin. By this definition, it would reflect primarily impaired insulin-stimulated glucose transport into cells that express the glucose transporter GLUT4 (skeletal muscle, heart muscle, and adipocytes).

Sustained hyperglycemia causes insulin resistance in humans (34) and in animal models (27), which leads to the concept of glucose toxicity. It accounts for the insulin resistance observed in patients with uncontrolled type 1 diabetes, which is reversible with insulin therapy (34). Similarly, sustained elevations of circulating nonesterified fatty acids also cause insulin resistance (lipotoxicity). Thus insulin resistance may be the cells' answer to the provision of excess nutrients. Several investigators (1, 20, 28) have proposed that increased flux through the hexosamine synthesis pathway (HSP) may function as a cellular nutrient sensor and play a role in the development of insulin resistance and the complications of diabetes. The role of HSP in the development of insulin resistance was first proposed by Marshall et al. (19) in 1991 and was based on studies in isolated rat adipocytes. Ever since then, a relatively copious literature that has recently been reviewed (2) has developed on this subject.

The HSP is a minor branch of the glycolytic pathway; glucose entry into HSP is catalyzed by the first and rate-limiting enzyme glutamine:fructose-6-phosphate (F-6-*P*) amidotransferase (GFAT), which converts F-6-*P* and glutamine into glucosamine 6-phosphate (GlcN-6-*P*) and glutamate. GlcN-6-*P* is metabolized to UDP-*N*-acetylglucosamine (UDP-GlcNAc), the major product of the pathway (19). UDP-GlcNAc and other amino sugars generated by the pathway provide building blocks of glycosyl side chains for proteins and lipids. UDP-GlcNAc is also the obligatory substrate of *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) transferase (OGT), a cytosolic and nuclear enzyme that modifies Ser/Thr residues of certain proteins by attaching single GlcNAc moieties in *O*-linkage (17, 18). *O*-GlcNAcylation frequently occurs on transcription factors and often involves known phosphorylation sites, suggesting a regulatory role (3). The process is reversible; *O*-GlcNAc is removed by a specific enzyme, *O*-GlcNAcase (32).

The synergistic effects of preincubation in high glucose and insulin on the development of insulin-resistant glucose transport in primary adipocytes were first reported by Garvey et al. (10). This model has been widely used to study the mechanisms of glucose-induced insulin resistance. We (23–25) have characterized it in some detail in 3T3-L1 adipocytes. Briefly, preincubation (18 h) in high (25 mM) glucose causes down-regulation of subsequent, acutely insulin-stimulated glucose transport, provided that low-dose (0.6 nM) insulin is included during preincubation. Preincubation in low (5 mM) glucose with insulin, or with high glucose without insulin, does not mimic the effect. The expression of glucose transporters (GLUT4 and GLUT1) is unaffected, and so is the proximal

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insulin-signaling cascade, as judged by inulin receptor substrate-1-associated phosphatidylinositol 3-kinase activity (23). However, both chronic exposure to low-dose insulin and high glucose independently impair acute insulin activation of Akt, and the two effects are synergistic (25). Since the effects of excess glucose flux via the HSP could be mediated by excess *O*-GlcNAc modification of some signaling protein(s) (21, 30, 31), we felt that overexpression of *O*-GlcNAcase in 3T3-L1 adipocytes, before they are submitted to conditions that promote insulin resistance, or inhibition of OGT by RNA interference (RNAi) would serve to test this hypothesis.

MATERIALS AND METHODS

Adenovirus preparation. pcDNA3.1His *O*-GlcNAcase (a generous gift from Dr. G. W. Hart) was digested with *Not*I/*Xba*1 and ligated into pAdTrack.CMV (19, 20). Plasmids were prepared using Plasmid Mini and Maxi Kits (Qiagen). Homologous recombination with pAdEasy-1 was performed in *Escherichia coli* BJ5183 cells (Stratagene). Recombinants were selected and transformed into *E. coli* XL-1 Blue (Stratagene), and large-scale plasmid preparations were generated as above. An empty vector preparation without the *O*-GlcNAcase insert was prepared in parallel. The adenovirus coexpresses green fluorescent protein (GFP). Adenovirus generation and amplification were performed in human embryonic kidney 293 cells as described (13, 14) and were purified using BD Adeno-X virus purification kit (BD BioSciences). Adenovirus concentration was determined by limiting dilution plaque assay and by spectrophotometric measurement.

To assess whether facilitated targeting of *O*-GlcNAcase into the nucleus affects insulin sensitivity, an *O*-GlcNAcase cDNA construct was prepared with a COOH-terminal nuclear targeting sequence and a c-myc tag for the generation of recombinant adenovirus. Human *O*-GlcNAcase was cut out of the pAdTrack vector with *Not*I and *Hind*II. The pShooter pCMV/nuc/myc vector (Invitrogen) containing three repeats of the nuclear localization signal (DPKKKRKV) and a c-myc tag (EQKLISEEDL) was linearized with *Not*I and filled in prior to cutting with *Xba*I. The *O*-GlcNAcase and the nuclear targeting sequence/c-myc tag were then ligated into the *Not*I/*Xba*I sites of pBluescriptSK-. Using site-directed mutagenesis (Quickchange; Stratagene) and the following primer, the stop codon between *O*-GlcNAcase and the nuclear targeting sequence was replaced with alanine, 5'-GGTCGGAGCCTGGCGGCCTTGTTGGCCGC-3'. The insert was then cut out of pBluescript SK- with *Not*I/*Xba*I/*Ssp*I, and the 2,694-bp fragment was ligated into the *Not*I/*Xba*I sites of the pAdTrack vector. The *O*-GlcNAcase/nuc/myc pAdTrack vector was linearized with *Pme*I and electroporated into BJ5183-AD-1 electrocompetent cells (Stratagene), following the protocol supplied by the manufacturer. Production of the virus was performed as described above, following the protocol of He et al. (13).

Viral infection of 3T3-L1 adipocytes and glucose transport assay. 3T3-L1 fibroblasts were differentiated into adipocytes as described (23). On *day 6* of the differentiation protocol they were infected with 2 × 10⁸ pfu/ml (MOI 200) empty or *O*-GlcNAcase adenovirus in serum-free DMEM for 4 h at 37°C followed by addition of FCS (to 10%) and incubation overnight. Fresh growth medium was applied. Five days after infection (~90% of cells expressing GFP), cells were incubated for 18 h in DMEM 1% FCS medium containing either 5 mM glucose or 25 mM glucose plus low-dose insulin (6 × 10⁻¹¹ to 6 × 10⁻¹⁰ M). Cells were serum and insulin deprived for 2 h and then stimulated or not for 15 min with either a low dose of insulin, which resulted in half-maximal stimulation of glucose transport (6 × 10⁻¹¹ M), or with a fully stimulating insulin dose (4 × 10⁻¹⁰ to 10⁻⁷ M), and glucose transport measured as the uptake of 2-deoxyglucose for 3 min, as previously described (23).

Total postnuclear extracts of cells were prepared in lysis buffer (50 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM sodium pyrophosphate, 100 μM sodium vanadate, 1% Triton X-100, and 10 μg/ml leupeptin, aprotinin, and pepstatin A, 1 mM phenylmethanesulfonyl fluoride, and 1 μM microcystin LR). Glucose transport was quantified by scintillation counting of a portion of the extract. Protein determination using Coomassie Protein Reagent (Pierce), *O*-GlcNAcase enzyme assays, and Western blots were also performed using these extracts. Nuclear extracts were prepared using the method of Dignam et al. (7).

RNAi. To reduce OGT expression, a small interfering RNA (siRNA) to a sequence in the COOH-terminal region of mouse OGT (Ref. NM-139144.2) corresponding to nucleotides 3420–3440 was transfected. This sequence was chosen on the basis of recent successful OGT suppression by an siRNA targeted against this region of rat OGT (11) that differs from the mouse in one nucleotide. The sequence of the siRNA against mouse OGT was sense 5'AGGGAACUA-GAUAACAUGCUU-3'. For the control, scrambled siRNA, the sequence was sense 5'-CGCAUUAAUCUAGUUCGCUUU-3'. A BLAST search of GenBank was carried out to avoid matches with other known sequences. Custom siRNA synthesis was carried out by Dharmacon (Lafayette, CO). This siRNA resulted in 50–60% suppression of OGT protein expression in 3T3-L1 adipocytes. To obtain a more complete suppression of OGT expression, we purchased SMARTpool Reagent from Dharmacon, which consists of a mixture of 4 unique siRNAs developed against the targeted gene and is guaranteed to silence the RNA by ≥75%. Transfection of this preparation decreased OGT protein expression by ~90% in 3T3-L1 adipocytes. For cells treated with nontargeting siRNA, we used Dharmacon's non-targeting siRNA pool of 4.

siRNA transfection or electroporation. 3T3-L1 adipocytes are difficult to transfect, and until recently, successful siRNA transfection was achieved only via electroporation (22). Recently, a technology using virus-derived amphipatic peptides has been developed (6) that directly interacts with nucleic acid cargos to form nanoparticles that diffuse through plasma membranes and release their cargos inside the cell. The technology has been adapted by the manufacturer (Genospectra, Fremont, CA) to several cell lines that are difficult to transfect, including 3T3-L1 adipocytes. We adopted the following protocol for transfection of the latter.

On *day 6* after initiation of the differentiation protocol, cells were subcultured to a density of 2.5 × 10⁴ cells/cm² and incubated overnight in DMEM containing 25 mM glucose and 10% FCS. Then, FCS was removed and cells were washed with PBS and transfected with a final concentration of 40 nM nontargeting or OGT siRNA using the Express-si Delivery Kit (Genospectra). Following a 4-h incubation with the siRNA, FCS was added to a concentration of 10%, and cells were incubated for an additional 18 h. Medium was replaced with fresh DMEM containing 25 mM glucose and 10% FCS for 24 h. Cells were then incubated for 18 h in DMEM containing 1% FCS and 5 mM glucose or 25 mM glucose plus 0.6 nM insulin and then serum and insulin deprived for 2 h and glucose transport measured as described.

For electroporation, on *day 6* after initiation of the differentiation protocol, 7 × 10⁶ cells were trypsinized, washed, and resuspended in PBS and electroporated with 20 nmol of scrambled or OGT siRNA, using the Gene Pulser XCell (Biorad) at a setting of 975 μF, 180 V, exponential decay in a 0.4-mm gap cuvette. Following electroporation, DMEM containing 25 mM glucose and 10% FCS was added to the cells, and they were plated at a density of 1.75 × 10⁶ cells/cm². Medium was refreshed the following day. Forty-eight hours after electroporation, cells were incubated in DMEM containing 1% FCS and 5 mM glucose or 25 mM glucose plus 0.6 nM insulin for 18 h and then serum and insulin deprived for 2 h and glucose transport measured as described.

Western blots of phosphorylated Akt, O-GlcNAcase, OGT, and O-GlcNAc-modified proteins. Cell extracts (20 μg protein) were separated on 7% SDS-PAGE and transferred to nitrocellulose. For

Western blot of phosphorylated (p)-Akt, O-GlcNAcase, and OGT, membranes were blocked for 1 h and then incubated overnight in 50 mM Tris, pH 7.4, 150 mM NaCl, 0.05% Tween 20, 5% nonfat dry milk (blocking buffer) containing anti-p-Akt rabbit polyclonal antibody (1:1,000, Cell Signaling), or anti-O-GlcNAcase or anti-OGT rabbit polyclonal antibody (1:5,000 and 1:2,000 respectively, both gifts of Dr. G. W. Hart). Following washing, membranes were incubated in goat anti-rabbit IgG (1:10,000, Jackson Immunoresearch) for 1 h, washed, developed with West Pico ECL Reagent (Pierce), exposed to film, and quantified by photodensitometry using a National Institutes of Health Image Analyzer.

For detection of O-GlcNAc-modified proteins, membranes were processed using the O-GlcNAc Western blot detection kit (Pierce), which uses the 110.6 antibody, developed by Comer et al. (4). In some experiments an alternative anti-O-GlcNAc antibody (RL-2, which was originally discovered as recognizing nuclear pore proteins), obtained from Affinity Bioreagents, was used.

O-GlcNAcase assay (modified from Refs. 8 and 9). Cell extracts (100 μl) were incubated for 30 min at 37°C in a final volume of 200 μl containing 50 mM sodium cacodylate, pH 6.4, 0.3% BSA, 2 mM p-nitrophenyl N-acetyl-β-D-glucosaminide (p-NPAGA), and 50 mM N-acetylgalactosamine without or with an O-GlcNAcase inhibitor, O-(2-acetamido-2-deoxy-D-glucopyranosylidene)-amino-N-phenyl-carbamate [PUGNAC (1 μM); Toronto Research Chemicals], which served as background. Reactions were stopped by the addition of 1.8 ml of 50 mM sodium carbonate, and A_{400} was determined (ϵ^{mM} = 17.7). O-GlcNAcase activity was determined by subtracting the +PUGNAC blank and expressed as micromoles of p-NPAGA per milligram extract protein per minute.

Materials. Unless otherwise indicated in the text, materials were purchased from Sigma.
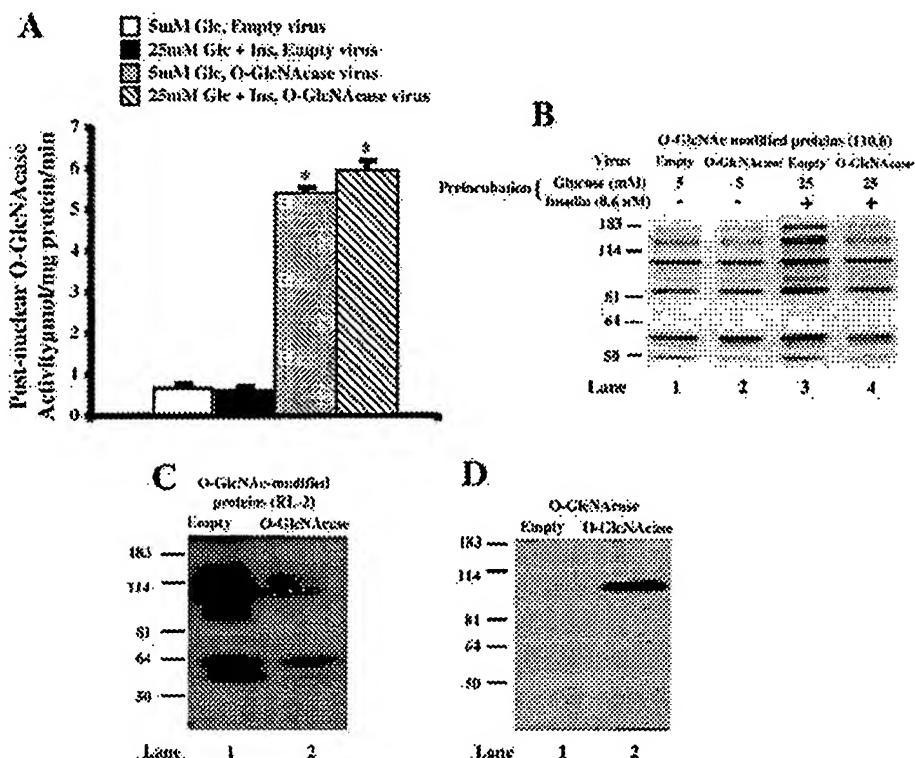
RESULTS

3T3-L1 adipocytes were infected with the O-GlcNAcase adenovirus or with empty virus on *day 6* of the differentiation

protocol, as described in MATERIALS AND METHODS. The time of infection was chosen because cells were easier to infect before they were fully differentiated. At the time of the experiment (5 days postinfection), >95% of the cells expressed the full adipocyte phenotype and >90% expressed GFP, indicating viral infection. On inspection under the microscope there were no obvious morphological differences between cells infected with empty or O-GlcNAcase-expressing adenovirus or noninfected cells.

To assess the level of expression of O-GlcNAcase, the enzyme activity was measured in postnuclear supernatants. The enzyme activity was increased >10-fold in cell extracts prepared from cells infected with O-GlcNAcase virus compared with cells expressing the empty virus. The conditions of preincubation of the cells (low or high glucose) made no difference (Fig. 1A). Figure 1B shows Western blots of postnuclear supernatants prepared from cells, as shown in Fig. 3, and developed with an antibody that specifically recognizes O-GlcNAc-modified proteins [antibody 110.6 (Ref. 4)]. Cells that were infected with empty virus and preincubated in high glucose showed clearly increased O-GlcNac modification of numerous proteins (Fig. 1B, lane 3), and this was diminished in cells overexpressing the O-GlcNAcase virus (Fig. 1B, lane 4). Figure 1C again demonstrates the enzyme activity in cell extracts using another antibody, RL-2. In comparing cells preincubated in high glucose expressing empty or O-GlcNAcase adenovirus, the latter showed a marked reduction in O-GlcNAc-modified proteins. Cells that were infected with adenovirus expressing O-GlcNAcase showed a strongly reactive band on Western blot developed with O-GlcNAcase antibody; the antibody was not sensitive enough to detect endogenous O-GlcNAcase in total cell extracts of cells infected with empty virus (Fig. 1D). Taken together, the data indicate that

Fig. 1. O-GlcNAcase amount and activity are markedly increased in cells infected with O-GlcNAcase virus. A: O-GlcNAcase activity was measured in postnuclear supernatants of cell lysates infected with the adenovirus expressing the enzyme or cells infected with empty virus using a method modified from Gao et al. (9), as described in MATERIALS AND METHODS. Means + SE are shown; n = 16 observations from 4 separate experiments. B: Western blots prepared from postnuclear supernatants of cell lysates developed with an antibody (110.6, Pierce) that specifically recognizes O-GlcNAc-modified proteins. 3T3-L1 adipocytes, which had been incubated for 18 h with 25 mM glucose plus 0.6 nM insulin, show clearly increased O-GlcNAcylation of several protein bands if infected with empty virus. However, total O-linked N-acetylglucosamine (O-GlcNAc) modification is diminished in cells overexpressing OGlcNAcase. C: the same cells developed with another O-GlcNAc-specific antibody, RL-2 (Affinity Bioreagents). O-GlcNAcylation is reduced in cells overexpressing O-GlcNAcase. D: Western blot developed with an anti-O-GlcNAcase antibody of postnuclear supernatants prepared from cells preincubated in high glucose plus 0.6 nM insulin and infected with empty virus (lane 1) or with O-GlcNAcase virus (lane 2).

the cells infected with adenovirus strongly expressed O-Glc-NAcase and that the enzyme was functional.

Cells were incubated for 18 h in DMEM containing 1% FBS and either 5 mM glucose or 25 mM glucose plus low-dose insulin (6 × 10⁻¹¹ to 6 × 10⁻¹⁰M) . Cells were then deprived of FCS and insulin for 2 h, stimulated or not (basal) for 15 min with a half-maximally or maximally stimulating dose of insulin, before measuring glucose transport for 3 min. As shown in Fig. 2, pretreating cells with high glucose plus insulin down-regulated subsequent basal and insulin-stimulated glucose transport in response to both half-maximal and maximal insulin stimulation by ~40% ($P < 0.03$-$P < 0.001$). Insulin stimulation above basal (Δinsulin) was also significantly reduced at both acute insulin concentrations. However, there was no significant difference in downregulation between cells infected with O-GlcNAcase virus or the empty virus.

Since preincubation in high glucose plus low-dose insulin downregulates Akt activation by acute insulin (25), we next tested this parameter. Cells were preincubated in 5 mM glucose or in 25 mM glucose plus 6 × 10⁻¹⁰ M insulin, deprived of serum and insulin for 2 h, and then stimulated with 10⁻⁷ M insulin for 15 min. Figure 3 illustrates a Western blot developed with an antiphosphoserine Akt (p-Akt) antibody. In the basal state, the p-Akt signal was too weak for graphic illustration. After acute stimulation by insulin, there was a robust p-Akt signal that was approximately twice as strong in cells preincubated in low glucose as it was in cells exposed to high glucose plus insulin. However, overexpression of O-GlcNAcase did not protect the cells from downregulation, and there was no significant difference in the level of p-Akt between cells infected with empty virus and O-GlcNAcase virus.

Fig. 3. O-GlcNAcase overexpression fails to restore the impaired activation of Akt by acute insulin stimulation in cells preincubated in high glucose plus low-dose insulin. Cells were treated as described in Fig. 1. Postnuclear supernatants were prepared from detergent-treated cell lysates in the presence of phosphatase and protease inhibitors. Proteins were separated by SDS-PAGE and immunoblotted with a p-Akt antibody that recognizes pSer[473]. A: representative Western blot. Basal Akt phosphorylation was too low to quantify. The bands shown are from cells that were acutely stimulated with insulin (10⁻⁷ M). B: results from 4 experiments. Data are normalized to Akt phosphorylation after acute insulin stimulation in cells preincubated in 5 mM glucose and infected with empty virus. Means + SE are shown; $n = 4$. *Significantly different from corresponding cells preincubated in 5 mM glucose. The expression of O-GlcNAcase had no significant effect.
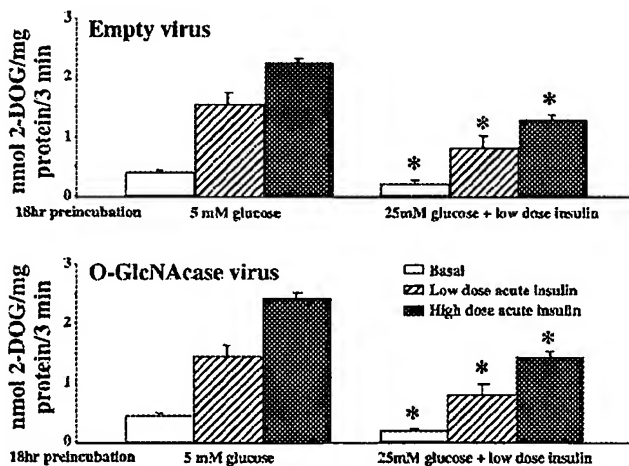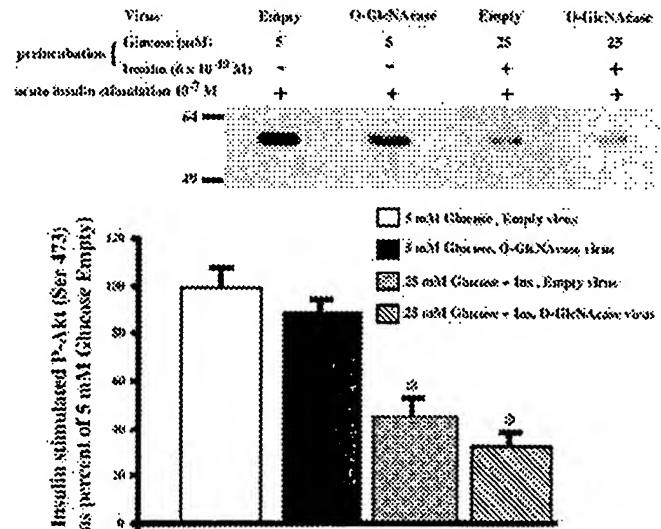

Fig. 2. Overexpression of O-GlcNAcase does not affect the development of insulin resistance in cells preexposed to high glucose plus low-dose insulin. 3T3-L1 adipocytes were infected with adenovirus expressing O-GlcNacase or with empty virus 5 days before experiments. More than 90% of cells expressed adenovirus, and 95% were fully differentiated at the time of the experiment. Cells were preincubated for 18 h with either 5 mM glucose or 25 mM glucose plus low-dose insulin (6 × 10⁻¹¹ to 6 × 10⁻¹⁰ M), serum and insulin deprived for 2 h, and then either not stimulated (basal) or stimulated for 15 min with insulin at a half-maximally or maximally stimulating dose. Glucose transport was then measured for 3 min [as 2-deoxyglucose (2-DOG) transport]. Means + SE are shown; $n = 4$–8. *Significantly decreased from corresponding observation in cells pretreated with 5 mM glucose, $P < 0.03$–0.001. There are no differences between cells treated with O-GlcNAcase or empty virus.

Since the development of insulin resistance most likely involves transcriptional regulation, it was important to determine the site of expression of the O-GlcNAcase enzyme. As shown in Fig. 4A, the overexpressed O-GlcNAcase was restricted to the cytosol, where its activity was markedly increased in cells infected with the O-GlcNAcase-containing virus. However, the activity in the nucleus was much lower than in the cytosol and was similar in cells infected with empty virus or with virus expressing O-GlcNAcase, suggesting that the overexpressed enzyme penetrates the nucleus poorly. This finding was further confirmed by examining Western blots (Fig. 4B) prepared from nuclear extracts or cytosol of cells treated under the different conditions and developed with an antibody (110.6) that specifically recognizes O-GlcNAc-modified proteins (4). In the cytosol and in the nucleus there was a trend toward increased O-GlcNAc modification of proteins in cells infected with empty virus and exposed to 25 mM glucose and low-dose insulin. Although cells expressing O-GlcNAcase tended to demonstrate less O-GlcNAcylation in the nucleus (compare lanes 2 and 4 in Fig. 4B), only in the cytosol was there a clear decrease in O-GlcNAc-modified proteins in cells overexpressing O-GlcNAcase after preincubation in low or in high glucose. (compare lane 6 with 8 and lane 5 with 7). The fact that transfected O-GlcNAcase is mainly restricted to the cytosol has previously been noted in COS-7 cells (9).

Our attempt at increasing nuclear targeting of O-GlcNAcase by expressing it with a nuclear localization signal was not successful. Although active enzyme was expressed in the cytosol, its nuclear expression did not improve. It also failed to
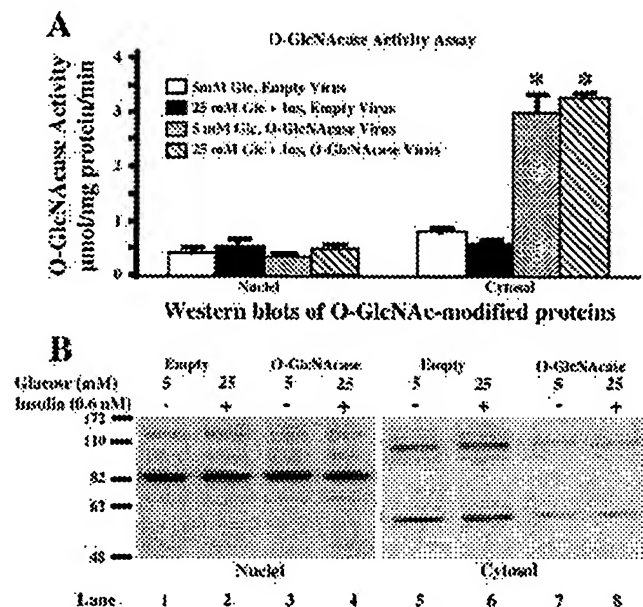
A

B

Fig. 4. Nuclear extracts show little evidence of increased O-GlcNAcase activity in cells infected with virus expressing the enzyme. Nuclear extracts were prepared as described in MATERIALS AND METHODS. The infranatant below the lipid layer overlying the nuclear pellet was designated cytosol. *A*: O-GlcNAcase activity measurements (as in Fig. 1A) in nuclear extracts and cytosol. Although the cytosol shows marked increases in O-GlcNAcase activity in cells infected with virus expressing the enzyme, there is no evidence of this in the nuclear extracts; $n = 3$ experiments. *Different from cells expressing empty virus, $P < 0.01$–0.001. *B*: Western blots of nuclear extracts or cytosol developed with an antibody (110.6 from Pierce) that specifically recognizes O-GlcNAc modified proteins. O-G, O-GlcNAcase-expressing virus. In cells infected with empty virus, there is a trend supporting increased glycosylation of proteins in cells preexposed to high glucose plus insulin. Although in the cytosol there is a marked decline in protein glycosylation in cells infected with O-GlcNAc-expressing virus, this trend is barely discernible in the nucleus (compare *lanes* 2 and 4). The gel is representative of 3 similar experiments.

have any effect on preventing the downregulation of the insulin response of glucose transport in cells preincubated in high glucose plus low-dose insulin (data not shown).

To rule out the possibility that the lack of effect of overexpressing O-GlcNAcase on the development of insulin resistance was due to some peculiarity of our adenoviral construct, we obtained an O-GlcNAcase-expressing adenovirus from Dr. W. H. Dillmann. Dr. Dillmann's group had successfully used this adenovirus to prevent the downregulation of sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) expression in cardiac myocytes incubated in high (25 mM) glucose (16). Results with this construct were essentially identical to those obtained with our O-GlcNAcase adenovirus. Although the activity was clearly overexpressed, it did not prevent the development of insulin resistance (data not shown).

Transfection or electroporation of OGT siRNA (developed to the COOH terminus) into 3T3-L1 adipocytes reduced the expression of OGT protein by 50–60% in 72 h, as assessed by Western blots. When the two methods were optimized, the results were very similar. However, overexpression of OGT siRNA again did not prevent the development of insulin resistance in cells preincubated in high glucose plus low-dose insulin. Preexposure to high glucose increased the O-GlcNAc modification of proteins, as assessed by 110.6 antibody stain-

ing, and this was decreased in cells transfected with OGT siRNA. The reduction of OGT expression was observed both in the cytosol and in nuclei, and results were similar upon introduction of siRNA by electroporation or by transfection (data not shown). Since these results may simply reflect that OGT was not sufficiently suppressed to mitigate the development of insulin resistance, we tested the SMARTpool Reagent (Dharmacon) consisting of a mixture of 4 siRNAs that are guaranteed to silence the desired RNA by $\geq 75\%$. In our case, OGT protein expression was decreased by ~90%, both in the cytosol and in nuclear extracts of 3T3-L1 adipocytes (Fig. 5A). Furthermore, the O-GlcNAc modification of proteins was markedly reduced both in the nuclei and in the cytosol of cells that had been treated with OGT siRNA (Fig. 5B). However, this did not affect either the basal or the insulin-stimulated glucose transport in cells preexposed to 5 mM glucose or to 25 mM glucose plus low-dose insulin and did not prevent or mitigate the development of insulin resistance in the latter (Fig. 5C). OGT knockdown also failed to restore the impaired insulin-stimulated Akt activation in insulin-resistant cells (Fig. 5D).

DISCUSSION

There is considerable evidence in the literature indicating that increased flux through the HSP, and specifically increased protein O-GlcNAcylation, can cause insulin resistance in adipocytes and in skeletal muscle (reviewed in Ref. 2). Mice overexpressing OGT in skeletal muscle and fat develop insulin resistance, as assessed by significantly reduced glucose disposal during hyperinsulinemic-euglycemic insulin clamp studies, as well as hyperleptinemia (21). Mice overexpressing the rate-limiting enzyme of the HSP, GFAT, in muscle and fat also develop insulin resistance (5, 15), which was evident in vivo (5, 15) but not in isolated muscle preparations and was attributed to cross talk between fat tissue and muscle (12). Obici (26) did not find evidence for insulin resistance, based on the hyperinsulinemic clamp technique, in mice overexpressing GFAT in skeletal muscle. Many authors base their conclusions on a link between insulin resistance and increased flux through the HSP on the observation that increased UDP-GlcNAc and insulin resistance tend to coexist, and the former enhances the rate of O-GlcNAc modification of proteins (2). Our observations do not negate the fact that increased protein O-GlcNAcylation can cause or contribute to insulin resistance of glucose transport and Akt activation; the experiments were designed to answer the question whether or not this process is required for its development.

There is strong evidence for a role of increased protein O-GlcNAc modification in the development of some of the complications of diabetes. In two cases, the techniques used here reversed the phenomenon; e.g., Dillmann reported that diabetic hearts showed increased protein O-GlcNAcylation as well as functional changes indicative of diabetic cardiomyopathy, such as contractile dysfunction and decreased expression of SERCA2a. All of these changes were reversed by treatment with O-GlcNAcase-expressing adenovirus (the adenovirus developed by Dillmann used the same plasmid construct as ours; both were gifts from G. W. Hart) (16). Similarly, exposure to high glucose induces plasminogen activator inhibitor-1 (PAI-1) expression in mesangial cells; this is mediated by enhanced
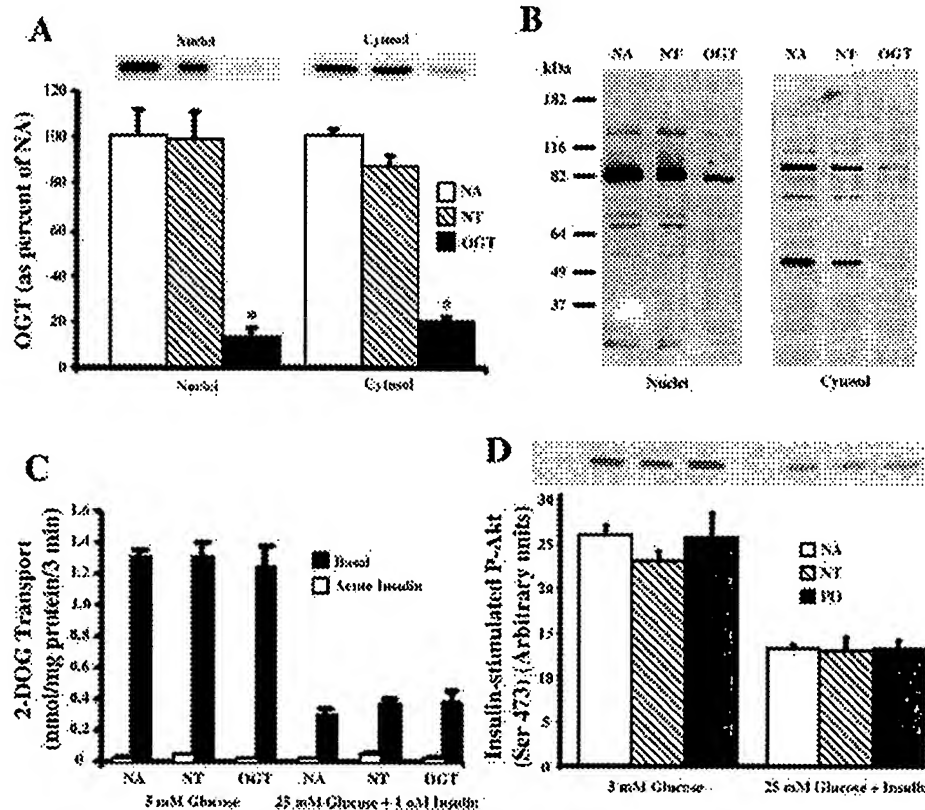
Fig. 5. Knockdown of O-GlcNAc transferase (OGT) does not prevent glucose/insulin-induced insulin resistance. In the experiments shown here, OGT knockdown was carried out using the "Smartpool Reagent"(Dharmacon), which consists of 4 unique siRNAs against mouse OGT; as nontargeting controls, we used Dharmacon's nontargeting siRNA pool of 4. Cells were transfected using a transfection reagent obtained from Genospectra, as described in MATERIALS AND METHODS, 48 h prior to initiating experiments and then cells were placed into conditions for 18 h, which will (or will not) cause insulin resistance. Thus cells were processed ~72 h after transfection. A: expression of OGT protein assessed by Western blots in nuclei and cytosol of cells that were incubated for 18 h in 25 mM glucose plus 0.6 nM insulin. NA, no RNA addition, although the cells underwent all the manipulations of the transfection protocol; NT, transfected with nontargeting siRNA; OGT, transfected with siRNA against OGT. Top of the graph shows a representative Western blot, whereas the bar graph (bottom) illustrates means + SE of 4 observations. *OGT < NA or NT, P < 0.001. B: representative Western blot of nuclear extracts and cytosol developed with the anti-O-GlcNAc antibody. The cells were transfected and preincubated under exactly the same conditions as in A, and the gel is representative of 4 similar experiments. Clearly, OGT knockdown markedly reduced protein O-GlcNAcylation both in the nucleus and in the cytosol. C: comparison of basal and maximally insulin-stimulated (0.1 uM) glucose transport between cells preincubated in 5 mM glucose, with cells preincubated in 25 mM glucose plus 0.6 nM insulin. High glucose/insulin caused insulin resistance, P < 0.01 vs. corresponding 5 mM glucose-treated cells. OGT knockdown had no effect; n = 4. D: same experimental design as C, except that insulin-stimulated Akt activation is studied, which is measured as the phosphorylation of Akt on Ser[473]. Basal phosphorylation of Akt in this system is barely detectable and is not shown. Cells preincubated in high glucose plus low insulin demonstrated impaired insulin-stimulated Akt activation, P < 0.01. OGT knockdown had no effect; n = 4.

O-GlcNAcylation and transcriptional activation of Sp1. Inhibition of O-GlcNAcylation by enhanced O-GlcNAcase expression via adenovirus, or by decreasing the expression of OGT via siRNA, inhibited high glucose-mediated PAI-1 induction, thus clearly demonstrating a causal connection between O-GlcNAcylation and specific transcriptional events (11). The complete lack of response to the marked diminution of O-GlcNAc protein modification in our studies may suggest that the insulin resistance of our model cannot be modified. This, however, is not the case. We have found that the insulin resistance of glucose transport and Akt activation is partially reversed by brief treatment with rapamycin or with an inhibitor of classical protein kinase C (unpublished observations).

The limited nuclear penetration of overexpressed O-GlcNAcase had been previously noted by Gao et al. (9) in Cos-7 cells. Since the development of insulin resistance most likely involves transcriptional events, we initially thought that the lack of effect of O-GlcNAcase treatment may reflect the insufficient

nuclear expression of the overexpressed enzyme. However, the experiments with OGT knockdown, where the enzyme was similarly decreased by ~90% in the nucleus and the cytosol, without affecting the development of insulin resistance, do not support this hypothesis. However, the limited nuclear transport of overexpressed O-GlcNAcase is of interest. Recent studies (29) have established that O-GlcNAcase is a bifunctional enzyme with activatable histone acetyl transferase and O-GlcNAcase activities. Furthermore, the enzyme associates strongly with OGT and accompanies it with histone deacetylases into transcription corepression complexes (33). These characteristics may be unfavorable for nuclear transport, at least in some cells. In cardiac myocytes (16) and in glomerular mesangial cells (11), O-GlcNacase penetrated the nucleus sufficiently to affect transcription.

In conclusion, overexpression of OGT in muscle and fat causes insulin resistance in vivo (21). However, the fact that two maneuvers that markedly reduced O-GlcNAcylation, in-

cluding reducing OGT expression by ~90%, failed to prevent or mitigate glucose/insulin-induced insulin resistance suggests that excess *O*-GlcNAcylation is only one of many causative factors of insulin resistance. Increased *O*-GlcNAcylation does not seem to be required for the development of insulin resistance of glucose transport and Akt activation in this model in 3T3-L1 adipocytes.

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